

Natural variation in nucleolar dominance reveals the relationship between nucleolus organizer chromatin topology and rRNA gene transcription in *Arabidopsis*

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In genetic hybrids, nucleolus formation on chromosomes inherited from only one parent is the epigenetic phenomenon, nucleolar dominance. By using *Arabidopsis suecica*, the allotetraploid hybrid of *Arabidopsis thaliana* and *Arabidopsis arenosa*, natural variation in nucleolar dominance was found to occur, providing a unique opportunity to examine homologous nucleolus organizer regions (NORs) in their active and inactive states. In *A. suecica* strain LC1, NORs derived from *A. arenosa* are active, whereas *A. thaliana*-derived NORs are silenced. In *A. suecica* strain 9502, NORs of both parental species are active. When active, NORs are partially, but not fully, decondensed. Both active and inactive LC1 NORs colocalize with the nucleolus, contradicting the long-standing assumption that rRNA gene transcription drives nucleolus association. Collectively, these observations clarify the relationships among NOR chromatin topology, rRNA gene transcription, and NOR-nucleolus associations. *A. suecica* strains LC1 and 9502 have each lost one pair of *A. thaliana* NORs during evolution, and amplified fragment-length polymorphism analysis further indicates that these strains are genetically very similar. These data suggest that nucleolar dominance can result from subtle genetic or epigenetic variation but is not a trait fundamental to a given interspecies hybrid combination.

In genetic hybrids or allopolyploids, nucleoli often assemble at specific chromosomal loci of one parent but not the other. This phenomenon, known as nucleolar dominance (1–4), was initially discovered as a change in chromosome structure (5). At nucleolar organizer regions (NORs), the loci where nucleoli form during interphase (6, 7), and where genes encoding the precursor transcript for 18S, 5.8S, and 25S rRNA are tandemly arrayed (8–10), NOR-bearing chromosomes in pure species (nonhybrids) display thin “secondary constrictions” at metaphase (6, 7). Navashin noted that in numerous interspecies hybrids, only the chromosomes of one parent display these secondary constrictions (5). Nuclear run-on assays later suggested that transcription of only one parental set of rRNA genes is the molecular explanation for nucleolar dominance (11, 12). Presumably, transcription of rRNA genes during interphase somehow reduces their condensation at metaphase, thus explaining the appearance of secondary constrictions (13).

A recurrent feature of nucleolar dominance in both plants and animals is that the NORs of the same species are always silenced, independent of maternal or paternal effects. This finding suggests that fundamental differences dictate the dominant and repressed sets of rRNA genes in a hybrid, possibly because of species-specific differences in the rRNA gene–RNA polymerase I transcription systems of the progenitors (1, 14). In keeping with the expectation of silencing in only one direction, *Arabidopsis arenosa*-derived rRNA genes were shown to be transcriptionally dominant over *Arabidopsis thaliana*-derived rRNA genes, both in a natural *Arabidopsis suecica* strain and in newly formed *A. suecica*-like allotetraploid hybrids (15). However, we show here that nucleolar dominance is not a fundamental property of *A.*

suecica. Instead, *A. thaliana*-derived NORs can be either silenced or active in different natural strains of *A. suecica*. This discovery of natural variation in nucleolar dominance presented a unique opportunity to study the same NORs in an active or silenced state, an opportunity we exploited to deduce the relationships between NOR chromatin topology, transcriptional activity, and NOR localization relative to the nucleolus. We show that partial, but not complete, NOR decondensation is the cytogenetic manifestation of active rRNA gene transcription. Both active and inactive NORs colocalize with the nucleolus, indicating that nucleolar association is not a reliable indicator of rRNA gene activity. Our analyses also reveal the number of NORs in *A. arenosa* and *A. suecica*. We find that one pair of *A. thaliana* NORs has been lost in *A. suecica* strains that differ with respect to *A. thaliana* NOR silencing, and the same pair is lost in both, indicating that NOR loss does not explain the variation in NOR activity. Amplified fragment-length polymorphism (AFLP) analyses further indicate that the strains are genetically similar. Collectively, these studies indicate that nucleolar dominance is not a fundamental trait of *A. suecica* due to species-specific differences inherent in its progenitors, but it is likely to be dictated by more subtle allelic or epigenetic differences.

Materials and Methods

Plant Material. *A. suecica* strain LC1 is a laboratory strain derived from Sue-1, provided by L. Comai (16) and reportedly the same strain examined by Hanfstaengl *et al.* (17). The LC1 strain has undergone several generations of single-seed descent in the Pikaard laboratory but is likely to be identical with Sue-1. The original wild population from which Sue-1 and LC1 are derived is unclear, although all known populations of *A. suecica* are restricted to northern Europe. *A. suecica* laboratory strain 9502 was derived from a plant of accession 90-10-085-10 (originating in Finland). Laboratory strain 94-53 was derived from a plant of accession 94-53-30-94-00 (collected in a botanical garden in Goettingen, Germany; the original location of the wild population is unknown). Plants of accessions 90-10-085-10 and 94-53-30-94-00 were made available by Steve O'Kane, as was *A. arenosa* accession 3651 (originating in Poland) (18). *A. thaliana* ecotype No-0 (Nossen, originating in Germany) was obtained from the Arabidopsis Biological Resource Center (Columbus, OH). Plants used for cytogenetic examinations were grown in a

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Abbreviations: AFLP, amplified fragment-length polymorphism; FISH, fluorescence *in situ* hybridization; NOR, nucleolus organizer region; DAPI, 4',6'-diamidino-2-phenylindole hydrochloride; IGS, intergenic spacer.

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greenhouse (20-h photoperiod at $25 \pm 2^\circ\text{C}$). Roots for cytological preparations were fixed in ethanol/acetic acid (3:1 vol/vol) and stored at -20°C until use. Interphase nuclei and chromosome spreads were prepared according to ref. 19.

Fluorescence in Situ Hybridization (FISH) Probe Labeling. DNA clones used for FISH analyses were pARR20-1, containing a 180-bp *A. thaliana*-specific pericentromeric repeat (20); pAt2, which corresponds to *A. thaliana* rRNA gene intergenic spacer (IGS) sequences from -2590 to $+92$ relative to the transcription start site, $+1$ (21); and pCaIGS, a complete *A. arenosa* rRNA gene IGS that was amplified by PCR with 25S and 18S rRNA-coding sequence primers flanking the IGS (D. A. Hayworth and B. A. Schaal, GenBank accession no. AF177417). The rRNA gene probes were labeled with biotin-dUTP or digoxigenin-dUTP by using a nick translation kit and conditions recommended by the supplier (Roche Applied Science). The pericentromeric repeat probe was amplified from pARR20-1 by PCR with the primers 5'-ATCCTCTAGAGTCGACCTGCA-3' and 5'-TTCCAGT-CACGACGTTGTAA-3', an initial denaturation step for 4 min at 94°C , and 35 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 45 s. The resulting 180-bp PCR products were labeled with digoxigenin-dUTP.

FISH. FISH in cell spreads was performed according to Jones and Heslop-Harrison (19). The hybridization mixture contained 100–200 ng of each probe in 50% formamide/2× SSC/10% dextran sulfate/salmon sperm blocking DNA (10 $\mu\text{g}/\mu\text{l}$)/0.1% SDS. The mixture was heated for 10 min at 70°C and then incubated on ice for a minimum of 5 min. The chromosome preparations and hybridization mixture were then denatured at 75°C for 10 min on a hot plate. Hybridization was carried out overnight in a moist chamber at 37°C . Two posthybridization washes, 5 min each, were performed in 50% formamide/0.1× SSC at 42°C . Digoxigenin-labeled probes were detected with anti-digoxigenin-fluorescein (Roche Applied Science) and biotin-labeled probes with Cy3-streptavidin (Sigma) according to Jones and Heslop-Harrison (19). Chromosomes were counterstained with 4',6-diamidino-2-phenylindole hydrochloride (DAPI) in Citifluor antifade buffer (AF1; Agar Scientific, Stansted, U.K.). Epifluorescence microscopy (Zeiss Axioskop 2) images were obtained by using a Zeiss AxioCam digital camera. Confocal FISH analyses used root-tips prepared according to ref. 34, propidium iodide counterstaining, and imaging with a MRC-600 confocal scanning laser microscope (Bio-Rad). Digital images were composed by using PHOTOSHOP (Adobe Systems, Mountain View, CA).

DNA Isolation. Nucleic acids of *A. suecica* laboratory strains LC1, 9502, and 94-53 were isolated from leaf tissue frozen in liquid nitrogen, ground to a powder, and mixed vigorously with 3 vol (wt/vol) of extraction buffer (250 mM Tris-HCl, pH 8.5/375 mM NaCl/25 mM EDTA/1% SDS/1% 2-mercaptoethanol/0.5 mg/ml heparin) in a 15-ml snap-cap tube. The resulting homogenate was subjected to centrifugation at $3,000 \times g$ for 10 min to pellet insoluble debris. The aqueous phase was extracted twice with phenol/chloroform, and total nucleic acids were precipitated by addition of 2 vol of ethanol. After centrifugation, pellets were resuspended in sterile water and large RNAs were precipitated with 3 M LiCl. Genomic DNA in the supernatant was recovered by ethanol precipitation and purified further by using a GeneClean Turbo Kit (BIO 101 Systems, Oligene, Carlsbad, CA).

RNA Isolation and S1 Nuclease Protection Assay. Twenty micrograms of LiCl-precipitated RNA was hybridized with oligonucleotide probes corresponding to the non-RNA (antisense) strand of *A. thaliana* or *A. arenosa* rRNA genes. Probes

spanned the transcription start sites and were 5' end-labeled by using T4 polynucleotide kinase (New England Biolabs) and [γ - ^{32}P]ATP. The sequence of the *A. thaliana*-specific probe was 5'-GGGTTCCCCACGGACTGCCAGACTCCC-TCAACACCCACCCCCCTATATAGCTGCC-3'; the *A. arenosa*-specific probe was 5'-GGAACCGAGTAGGGAG-GTACCCTCGGTCTGCCAGACTTCACCAACACCCAC-CCCCTATATAGCTTTTT-3'. After an initial denaturation step at 99°C for 15 min, probe-RNA hybridization reactions were incubated overnight at 50°C . Probe-RNA hybrids were then subjected to S1 nuclease (Invitrogen) digestion (750 units/ml) at 50°C for 45 min. Resulting digestion products were resolved on a urea/10% polyacrylamide sequencing gel. Gels were dried onto filter paper and exposed to x-ray film.

AFLP Analysis. AFLP assessment was conducted by using the AFLP Analysis System II with adapters, primers, and protocols provided by the supplier (GIBCO). In brief, 250 ng of purified genomic DNA was digested to completion by using the restriction endonucleases *Eco*RI and *Mse*I. Digested DNAs were then ligated to *Eco*RI and *Mse*I adapters, and the resulting ligation products were amplified by PCR with primers matching the adapters. Resulting PCR products provided the templates for subsequent PCR that reduced the complexity of the DNA fragment pool by using selective primers. These latter selective PCR used an *Eco*RI-AC primer (*Eco*RI adapter primer extended on the 3' end with an adenosine and a cytosine), 5' end-labeled by using T4 polynucleotide kinase and [γ - ^{32}P]ATP, together with one of several primers specific to the *Mse*I end of the fragments (M-CAA, M-CAC, M-CAT, M-CTC, M-CTA, and M-CTT). Each primer pair amplifies a distinct subset of DNA fragments in the template mix. Resulting PCR fragments were resolved on a 7.5 M urea/6% polyacrylamide (19:1 acrylamide/bisacrylamide) sequencing gel. The gel was vacuum dried onto filter paper and exposed to x-ray film for 24–48 h.

Results

Origins of NORs in *A. suecica*. Although *A. thaliana* NORs have been well characterized cytogenetically, physically, and genetically in the ecotypes Columbia and Landsberg erecta (23–31), the numbers and locations of NORs in *A. arenosa*, *A. suecica*, and most ecotypes of *A. thaliana* are unknown. Using *A. thaliana* and *A. arenosa* rRNA gene IGS sequences as FISH probes, we examined the chromosomes of *A. suecica* and its progenitors (Figs. 1 and 2). The genomes of *A. suecica* strains LC1 and 9502 include a transposable element found in *A. thaliana* ecotype No-0 but missing from most ecotypes of *A. thaliana*, suggesting that No-0 may be related to one progenitor of LC1 and 9502 (32). In *A. thaliana* ecotype No-0, four NOR FISH signals are observed among the 10 chromosomes in diploid root-tip cells (Fig. 1 *A* and *B*). These signals are presumed to correspond to *NOR2* and *NOR4* (two of each NOR in a diploid cell), which are located adjacent to the telomeres at the tops of chromosomes 2 and 4 in the ecotypes Columbia and Landsberg erecta (26, 28). *A. arenosa* was found to have 12 NORs among its 32 chromosomes (Fig. 1 *C* and *D*). Because many *Arabidopsis* species have 16 chromosomes per diploid genome (33), *A. arenosa* is presumed to be a tetraploid ($2n = 4x = 32$, where n equals the chromosome number in gametes and x represents the fundamental chromosome number). Hence, *A. arenosa* has three NORs per 1x complement of chromosomes, whereas *A. thaliana* has only two.

A. suecica has 26 chromosomes, as seen by DAPI staining in LC1 and 9502 (Fig. 2 *B* and *D*), the expected number for an allotetraploid (amphidiploid) possessing a 2x genome complement from both *A. thaliana* (10 chromosomes) and *A. arenosa* (16 chromosomes). FISH analysis using a probe corresponding to *A. thaliana* 180-bp centromere repeats confirms that 10 of the

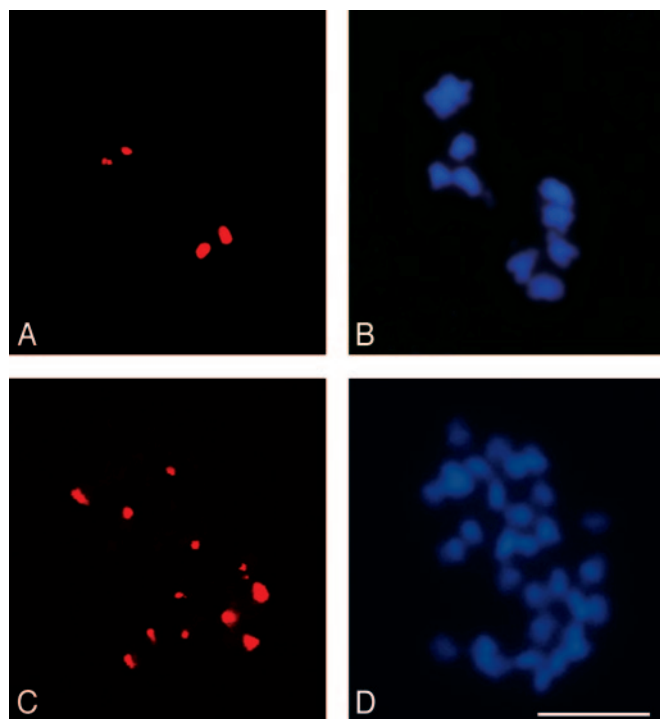


Fig. 1. FISH to mitotic metaphase cells from *A. thaliana* ecotype No-0 (A and B) and *A. arenosa* strain 3651 (C and D). An *A. thaliana*-specific rRNA gene IGS probe (pAt2) was used in A, whereas an *A. arenosa* IGS probe (pCalGS) was used in C (red FISH signals). DAPI staining shows 10 chromosomes for *A. thaliana* (B) and 32 for *A. arenosa* (D). (Bar = 5 μ m.)

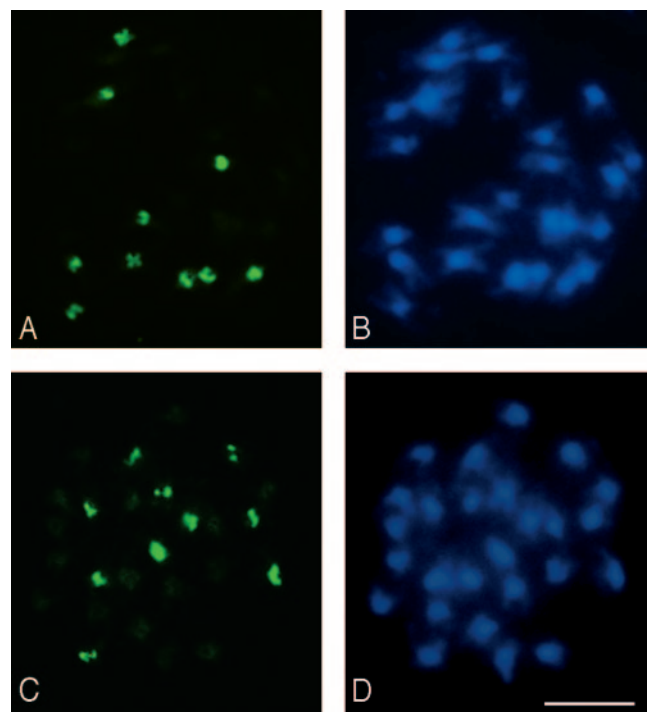


Fig. 2. Metaphase cells of *A. suecica* strains LC1 (A and B) and 9502 (C and D) after *in situ* hybridization with an *A. thaliana*-specific centromere probe (pARR20-1). Ten FISH signals (green), the diploid *A. thaliana* ($2n = 2x = 10$) number, are observed in both strains (A and C). Sixteen unlabeled chromosomes visualized with DAPI (B and D) correspond to the expected $2x$ *A. arenosa* ($2n = 4x = 32$) chromosome number. (Bar = 5 μ m.)

chromosomes in *A. suecica* are of *A. thaliana* origin (Fig. 2 A and C).

Although *A. suecica* strains LC1 and 9502 have the expected diploid number of *A. thaliana* chromosomes, they only have two *A. thaliana*-like NORs (Fig. 3 A and C, red FISH signals) rather than four, the expected diploid number. By contrast, six *A. arenosa*-derived NORs are present in both *A. suecica* strains (Fig. 3 A and C, green FISH signals), matching the expected number for a $2x$ complement of *A. arenosa* chromosomes. These results show that one pair of NORs of *A. thaliana* origin has been lost during evolution in both *A. suecica* strains examined. Based on linkage to cytogenetic and molecular markers, the *A. thaliana* NOR pair that has been retained in *A. suecica* is *NOR4* (O.P., N.N., M.S., R.J.L., M. S. Lewis, C.S.P., and W.V., unpublished data).

Natural Variation in Nucleolar Dominance Revealed by Transcription

Analyses. Transcription of *A. thaliana*- and *A. arenosa*-derived rRNA genes in *A. suecica* strains LC1, 9502, and 94-53 was examined by using the S1 nuclease protection assay to determine steady-state transcript levels (Fig. 4). Total RNA isolated from each strain was split into aliquots and hybridized in separate reactions to radioactively labeled oligonucleotide probes that were specific for *A. thaliana* or *A. arenosa* rRNA transcripts initiated at the correct promoter start site (defined as +1). After digestion of the resulting RNA–DNA hybrids by S1 nuclease, the digestion products were subjected to denaturing PAGE and autoradiography. The *A. thaliana* probe detects *A. thaliana* rRNA gene transcripts but not *A. arenosa* transcripts (compare lanes 1 and 4 in Fig. 4). The *A. arenosa* probe is equally species-specific in control reactions (compare lanes 2 and 3 in Fig. 4). In *A. suecica* strains LC1 and 94-53, transcripts from the *A. arenosa* rRNA genes are abundant (lanes 6 and 8), but *A. thaliana* rRNA gene transcripts are

undetectable by S1 nuclease protection (lanes 5 and 7). *A. thaliana* rRNA gene transcripts are readily detected in LC1 if plants are treated with chemical inhibitors of cytosine meth-

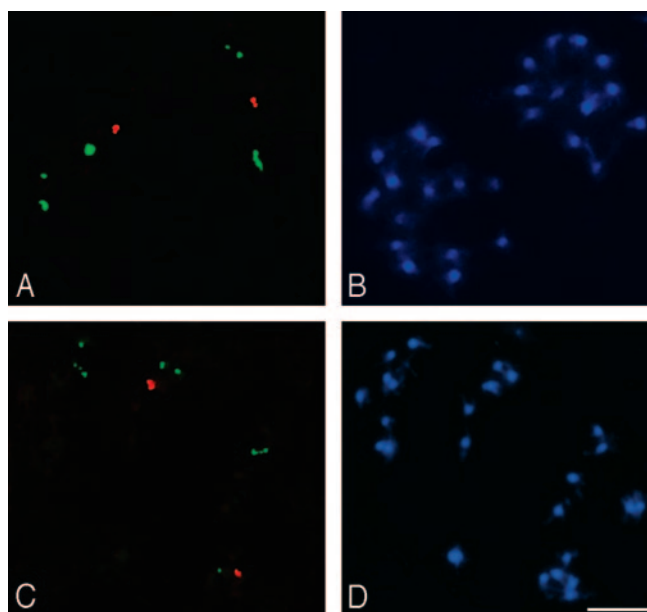


Fig. 3. Metaphase *A. suecica* LC1 cells (A and B) and 9502 (C and D) after *in situ* hybridization with rRNA gene IGS probes pAt2 (from *A. thaliana*, in red) and pCalGS (from *A. arenosa*, in green). In both strains of *A. suecica* only one pair of *A. thaliana* NORs is present, whereas the expected six NORs from *A. arenosa* are retained. Chromosomes were counterstained with DAPI in B and D. (Bar = 5 μ m.)

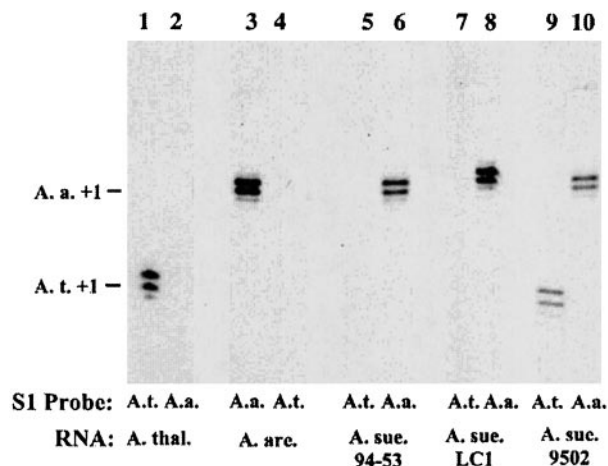


Fig. 4. Natural variation in nucleolar dominance in *A. suecica*. RNA isolated from three strains of *A. suecica* (*A. sue.*), *A. thaliana* (*A. thal.*), and *A. arenosa* (*A. are.*) was tested for *A. thaliana* or *A. arenosa* pre-rRNA transcripts by using an S1 nuclease protection assay. S1 probes were specific for *A. thaliana* (*A.t.* probe; lanes 1, 4, 5, 7, and 9) or *A. arenosa* (*A.a.* probe; lanes 2, 3, 6, 8, and 10) rRNA genes, as shown by the controls in lanes 1–4. In *A. suecica* strain 9502, *A. arenosa* and *A. thaliana* rRNA transcripts are equally abundant, indicating codominance.

ylation or histone deacetylation (ref. 15; R.J.L. and C.S.P., unpublished data), indicating that the lack of *A. thaliana* transcript signal is due to rRNA gene silencing and not to probe–RNA transcript incompatibilities. rRNA transcripts of both *A. thaliana* and *A. arenosa* are detected in *A. suecica* strain 9502 (Fig. 4, lanes 9 and 10), which is true in all individuals studied over multiple generations (data not shown). These data indicate that nucleolar dominance does not occur in all *A. suecica* strains but exhibits natural variation as a trait.

Variation in NOR Chromatin Topology Correlates with Variation in Gene Expression. Variation in nucleolar dominance in *A. suecica* strains LC1 and 9502 provided a unique opportunity to compare homologous NORs in their active and inactive states, an evaluation not otherwise possible. These examinations revealed distinct differences in the NOR chromatin organization in interphase nuclei of LC1 and 9502 (Fig. 5). In LC1, *A. arenosa*-like NORs show partial decondensation at interphase, which is manifest in two ways. One manifestation is the appearance of FISH signal spots in excess of the number of NORs because of decondensation in central regions of the NORs flanked by regions that remain condensed (Fig. 5B; red signals). The other manifestation of decondensation is reduced FISH-staining intensity overall compared with condensed mitotic NORs (compare red signals in Fig. 5B with the green signals in Fig. 3A). By contrast to *A. arenosa*-derived NORs, the two *A. thaliana* NORs in *A. suecica* strain LC1 are consistently condensed into two discrete knobs (Fig. 5A and B, green signals) that dwarf the red *A. arenosa* NOR signals because of the decondensation of the latter. This FISH signal differential is not apparent at mitosis when both dominant and underdominant NORs are more condensed (compare red and green signals in Figs. 5B and 3A).

Unlike *A. suecica* strain LC1, the *A. thaliana*-like NORs in interphase nuclei of *A. suecica* strain 9502 are partially decondensed, resulting in four FISH signals due to decondensation of central regions of the NORs (Fig. 5D and E, red signals). On examining ≈ 120 cells in each strain (LC1 and 9502), scoring the number of *A. thaliana* NOR FISH signals per cell, and comparing the resulting distributions, strain 9502 shows a significant increase in NOR decondensation relative to strain LC1 (Stu-

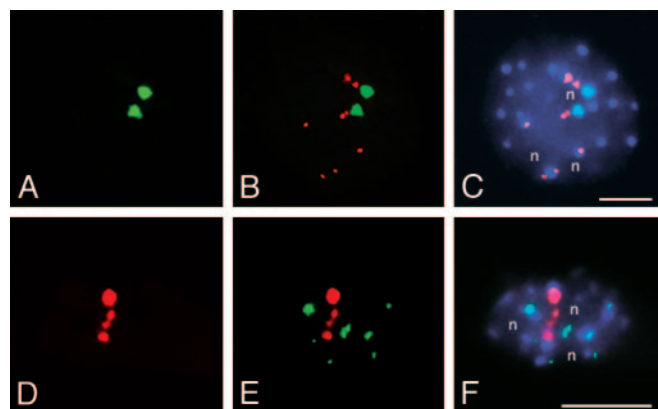


Fig. 5. Root-tip interphase cells of *A. suecica* strains LC1 (A–C) and 9502 (D–F) after *in situ* hybridization with rRNA gene IGS probes pAt2 (from *A. thaliana*) and pCalGS (from *A. arenosa*). (A–C) A meristematic interphase nucleus from strain LC1 shows two condensed *A. thaliana*-derived NORs (green signals) and partially decondensed *A. arenosa* NORs (red signals). (D–F) A meristematic interphase nucleus from strain 9502 shows *A. thaliana* NORs decondensed (red signals) and decondensed *A. arenosa* NORs (green signals). (C and F) FISH and DAPI signals are superimposed; n denotes nucleoli. (Bar = 5 μ m.)

dent's *t* test $P < 0.01$; data not shown). Combined with the S1 nuclease protection data of Fig. 4, these data indicate that partial NOR decondensation is a cytogenetic manifestation of active rRNA gene transcription at an NOR.

Inactive NORs Associate with the Nucleolus. After staining of interphase chromatin with DAPI or propidium iodide, nucleoli appear as dark, unstained regions (see Figs. 5 and 6). The inactive *A. thaliana*-derived NORs in *A. suecica* strain LC1 are consistently found in association with nucleoli, as is seen most clearly in Fig. 6B. As further evidence, Fig. 6C–E shows consecutive confocal sections of a single LC1 nucleus, confirming, in a third dimension, that *A. thaliana*-derived NORs localize

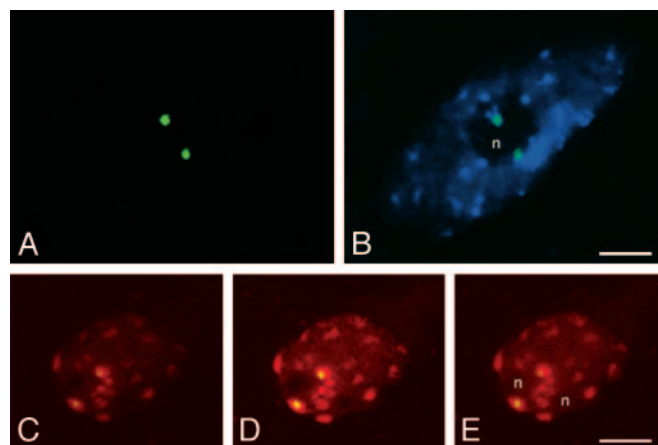


Fig. 6. Both active and inactive NORs associate with the nucleolus. (A and B) A differentiated root-tip cell from strain LC1 showing the two *A. thaliana*-derived NORs (green signals; pAt2 FISH probe) associated with the nucleolus (n), which appears as a dark region unstained by DAPI (FISH and DAPI signals are superimposed in B). (C–E) Three optical sections obtained by confocal microscopy of a meristematic root-tip cell of strain LC1, further demonstrating the association of inactive *A. thaliana*-derived NORs with nucleoli. *A. thaliana*-derived NORs appear yellow because of the superimposition of green FISH signals (pAt2 probe) and red, propidium iodide-stained chromatin. Note that heterochromatic regions provide the brightest red signals. NOR signals in C–E appear smaller than in A and B because they are individual confocal sections; integrated images of many sections would be larger. (Bar = 5 μ m.)

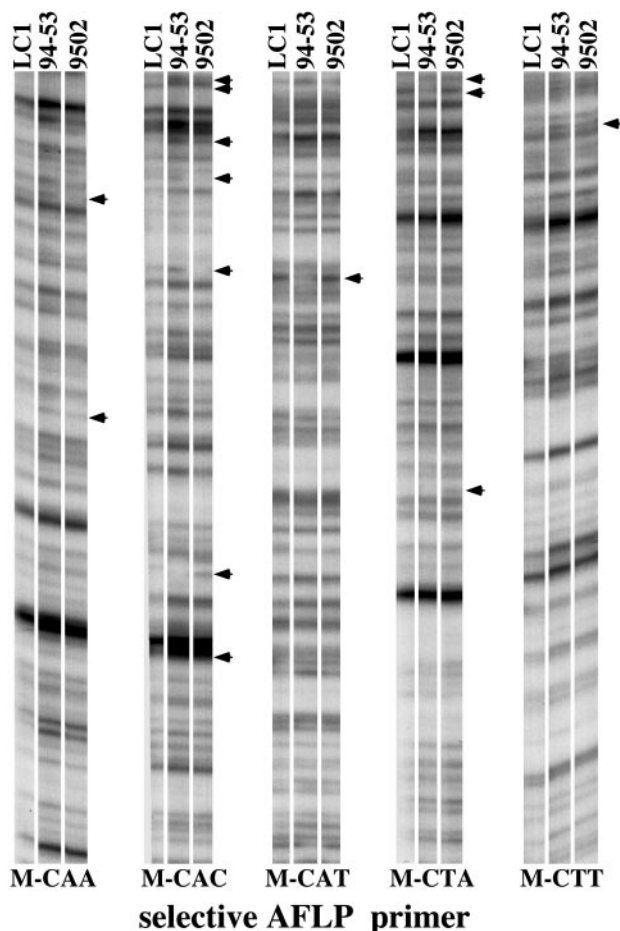


Fig. 7. AFLP analysis of *A. suecica* strains showing natural variation in nucleolar dominance. Genomic DNA fragments of *A. suecica* strains LC1, 94-53, and 9502 were analyzed by using five different selective primers (M-CAA, M-CAC, M-CAT, M-CTA, and M-CTT). Bands of distinct mobility that are not common to all three strains are marked by arrows. Bands that differ only in intensity are not marked because these might result only from differences in restriction endonuclease digestion efficiency, possibly due to cytosine methylation, an epigenetic modification.

to the periphery of the nucleolus. This association of inactive *A. thaliana*-derived NORs with the nucleolus in LC1 occurred in 84% of meristematic root-tip cells and 76% of differentiated root-tip cells (50 of each cell type examined). Collectively, our data suggest that nucleolar association cannot be taken as evidence of transcriptional activity of an NOR, in agreement with a careful study of NORs in cultured mammalian cells (22).

Genetic Similarity of *A. suecica* Strains Displaying Variation in Nucleolar Dominance. Our detection of natural variation in nucleolar dominance prompted us to evaluate the degree of genetic similarity in *A. suecica* strains 9502, LC1, and 94-53 by using AFLP analysis of genomic DNA (Fig. 7). By using five different primer pair combinations, AFLP indicated that all three strains of *A. suecica* are genetically very similar, with only $\approx 4\%$ (14 of 327) of all amplified bands revealing a polymorphism in any pairwise combination. These data suggest that variation in nucleolar dominance is likely to result from relatively subtle genetic or epigenetic variation.

Discussion

A previous study showed that nucleolar dominance occurs both in a natural strain of *A. suecica* and in synthetic *A. suecica*-like

allotetraploids created in the laboratory (15). In the current study, we show that nucleolar dominance is not a fundamental trait of *A. suecica* as a species. We have exploited this natural variation in nucleolar dominance to deduce the relationships among NOR chromatin topology, NOR–nucleolus association, and rRNA gene transcription. Our observations suggest that transcriptionally inactive NORs are highly condensed both at interphase and at metaphase and thus appear as bright spots after FISH. By contrast, active NORs appear to be composed of condensed knobs that are interconnected by decondensed regions of rRNA gene chromatin. One interpretation of these data is that only a subset of the rRNA genes is transcribed even at dominant NORs, and this subset is the fraction that makes up the decondensed NOR chromatin. Indeed, in wheat–rye chromosome addition lines, dominant wheat NORs also display condensed knobs interspersed with decondensed rRNA gene chromatin (A. P. Santos, M.S., N.N., and W.V., unpublished data). In these addition lines, only the decondensed regions of wheat NORs incorporate BrUTP, indicating that these intervals contain transcriptionally active rRNA genes. Likewise, investigations of diploid rye have shown that condensed rRNA gene chromatin is located at the periphery of the nucleolus, whereas decondensed chromatin extends into the central portions of the nucleolus where rRNA transcription takes place (34). Condensed regions are thus thought to be portions of the NOR in which transcriptionally inactive rRNA genes are packaged into heterochromatin.

Although it is difficult to quantify the number of rRNA genes located within the condensed and decondensed portions of the NOR, visual inspection of FISH images suggests that only a small fraction of the *A. thaliana* rRNA genes are decondensed, and are thus presumed to be transcriptionally active, in *A. suecica* strain 9502. This finding may not be surprising given the large number of rRNA genes in plants compared with budding yeast, *Drosophila*, *Xenopus*, or mammals, whose haploid rRNA gene numbers range between 150 and 400 genes. In yeast and mouse cells, psoralen-crosslinking experiments suggest that only one-third to one-half of the rRNA genes are transcribed (which makes them accessible to psoralen) in actively growing cultured cells (refs. 35–38; reviewed in ref. 39). *A. thaliana* has relatively few rRNA genes compared with other plants, yet still has an estimated 750 rRNA genes per haploid genome. If only 75–200 rRNA genes need to be transcriptionally active (extrapolating from yeast and mouse, respectively), one might expect only ≈ 10 –27% of the rRNA genes in a diploid *A. thaliana* nucleus to be active. In an allotetraploid hybrid such as *A. suecica* strain 9502, which transcribes rRNA genes of both parental species, the fraction of the *A. thaliana*-derived rRNA genes that are transcribed may be lower still. This finding may explain why the condensed knobs are the most prevalent feature of *A. thaliana*-derived NORs in *A. suecica* strain 9502, despite the transcription of *A. thaliana* rRNA genes in this strain.

As shown in Fig. 6, the *A. thaliana*-derived NORs associate with the nucleolus in *A. suecica* strain LC1 even though no transcription from these NORs can be detected by using the S1 nuclease protection assay. An interesting parallel in mammals is that transcriptionally inactive human NORs in mouse–human cell hybrids colocalize to the nucleolus with transcriptionally active mouse NORs (22). It is possible that trace levels of transcription play some role in NOR–nucleolus associations. Alternatively, positional cues other than rRNA gene transcription may be key. One possibility is that sequence-specific DNA-binding proteins, rather than rRNA transcription, provide signals for nucleolus assembly or NOR–nucleolus association.

The basis for natural variation in nucleolar dominance is not clear. Although *A. suecica* strains LC1 and 9502 differ in terms of nucleolar dominance, they are genetically similar. One large-scale similarity is that one pair of *A. thaliana*-derived NORs has

been lost in both LC1 and 9502, the missing NORs being *NOR2* in both cases (O.P., N.N., M.S., R.J.L., M. S. Lewis, C.S.P., and W.V., unpublished data). Likewise, the *A. thaliana*-derived NORs in both strains appear to be similar in size based on metaphase FISH signal intensity, are similarly positioned near the ends of their respective chromosomes, and are similarly associated with nucleoli regardless of transcriptional activity. On a finer scale, AFLP revealed that only $\approx 4\%$ of amplified bands showed any difference in size in the two strains. For comparison, AFLP analysis of 38 *A. thaliana* ecotypes revealed that, on average, $\approx 17\%$ of AFLP bands are polymorphic between ecotypes, with the most similar pair of ecotypes revealing polymorphic bands at a frequency of $\approx 4\%$ (40). Hence, the *A. suecica* strains we have examined are genetically very similar, comparable with the similarity of the most closely related ecotypes of *A. thaliana*. Collectively, these observations suggest that natural variation in nucleolar dominance in *A. suecica* is likely to result from subtle genetic (or epigenetic) variation rather than dramatic genome restructuring events. This finding suggests that it might be possible to identify different ecotypes of the progenitor species that program different nucleolar dominance outcomes when crossed to re-create the hybrid. If such variation can be found among *A. thaliana* parents, in particular, the genetic tools available could facilitate identification of genes controlling nucleolar dominance.

One clue concerning the types of regulatory genes that might explain natural variation in nucleolar dominance is the fact that

decondensation of *A. thaliana*-derived NORs in *A. suecica* strain 9502 (Fig. 5) is also accompanied by a greater degree of decondensation among the *A. arenosa*-derived NORs when compared with strain LC1. Whereas the average number of condensed FISH signals observed for the dominant *A. arenosa*-like NORs in 136 interphase cells of strain LC1 was 5.9 ± 1.7 (mean \pm SE), the average number observed in 120 interphase cells of strain 9502 was 8.7 ± 2.0 , a significant difference ($P < 0.01$; Student's *t* test). Examination of chromatin visualized by DAPI, which stains the condensed heterochromatin most brightly, failed to reveal obvious differences in overall chromatin condensation between the two strains. Therefore, one possibility is that strain 9502 has decreased activity for one or more proteins that affect NOR or rRNA gene condensation without acting throughout the whole genome. Future studies are needed to elucidate whether such changes in NOR chromatin topology are a cause or a consequence of changes in rRNA gene transcription.

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